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DIRECTEUR: Dr. J. G. TEN HOUTEN

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AN IMPROVED METHOD FOR MAKING THIN AGAR LAYERS IN POLYETHYLENE BAGS

DOOR

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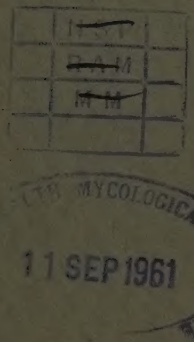

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AN IMPROVED METHOD FOR MAKING THIN AGAR LAYERS IN POLYETHYLENE BAGS

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The method for making thin agar layers in polyethylene bags (DEN OUDEN, 1958) has one major drawback. It is impossible to make them rapidly in large numbers and with a sufficient degree of sterility. Moreover plain nutrient agar appears not to be entirely satisfactory as a medium for prolonged growth. When the bags are manipulated, water is "excreted" by the agar (syneresis water) and nematodes tend to swim in this water and are not able to enter the agar. When plants have depleted the water from the agar, this cannot be replaced, as cold agar does not take up water.

IMPROVEMENT OF THE AGAR MEDIUM

A satisfactory medium for use in the polyethylene bags was obtained by adding methylcellulose to the agar. This medium does not "excrete" water so readily as pure agar and it absorbs water added after plants have consumed some. The complete medium is made by mixing one part of a 1% solution of methylcellulose in water (prepared some twelve hours before the mixing) with one part of 5% agar dissolved in a suitable nutrient solution (double concentration).

MAKING POLYETHYLENE BAGS

The bags for the agar layers are cut from polyethylene tube of appropriate width. Generally these tubes are almost sterile inside. If complete sterility is desired, the inside is moistened with a 0.2% solution of corrosive sublimate containing a wetting agent, for at least ten minutes and rinsed with sterile water. Before cutting, as much water and air as possible must be removed from the piece of tube. This is done after sealing one end and then pulling the tube gently over a sharp, straight edge, beginning close to the seal (fig. 3). When the water has been removed, seals are made at distances equal to the length of the bags wanted. These seals must be wide enough to be cut in half over their full length or two seals must be made close together (fig. 4). By cutting along the middle of a wide seal or between two seals, bags are obtained. If the tube is cut first and then sealed, contaminated air may slip in at the corners of the cut pieces.

APPARATUS FOR STERILIZING THE AGAR MIXTURE AND MAKING THE LAYERS IN THE BAGS

The agar mixture is put in a small autoclave or pressure cooker, which can withstand a pressure of 2 atmospheres. The vessel (fig. 1, A) is provided with a manometer (fig. 1, c), a safety valve (d), an inlet with air filter (B) (a metal tube densely packed with cotton wool) and an outlet with valve (b). There is also a valve between the air filter and the vessel to prevent agar from entering the former during the sterilisation (fig. 1, a) and one in the air inlet to the air

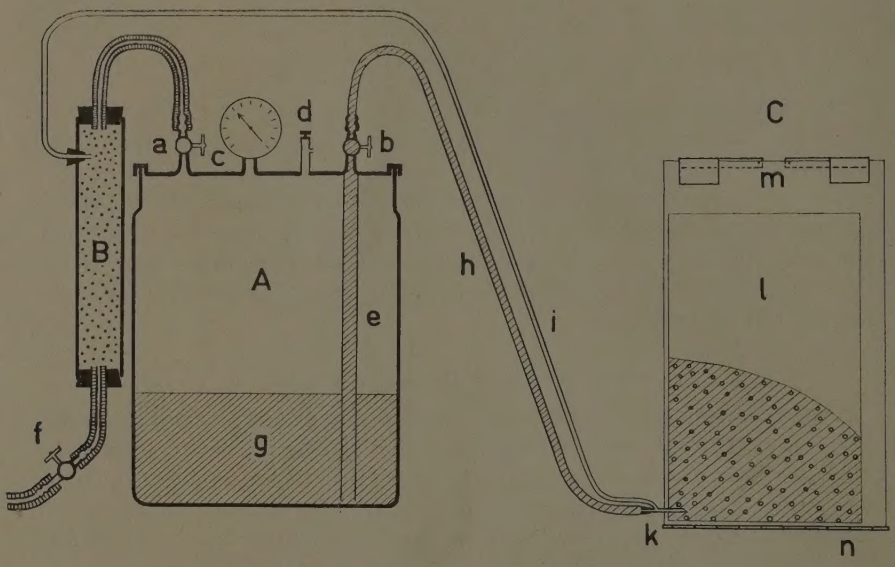

Fig. 1. Diagram of the apparatus, 1/6 natural size. A. Pressure vessel; B. Air-filter with cotton wool; C. Press to keep bags flat; a. valve for air inlet; b. valve for agar outlet; c. manometer; d. safety valve; e. tube for conducting agar to plastic bag; f. valve in pressure line; g. 2½% agar, ½% methylcellulose, nutrient solution; h. tube to injection apparatus; i. tube for injection of air; k. injection apparatus (see fig. 2); l. polyethylene bag; m. clamps for keeping perspex and metal sheet together; n. piano hinge.

filter (f). The outlet is provided with a rubber tube (h) fortified with linen tape to take a pressure of two atmospheres. From the air filter (B) air under pressure can be led through a thin rubber tube (i). To the tubes h and i an apparatus (fig. 1, k, fig. 2) is attached by which agar with small air bubbles can be led into a polyethylene bag (fig. 1, l). On tubes h and i pinchcocks are placed (fig. 2, s). These are connected in such a way that they open simultaneously (see fig. 5) so that agar can pass through h and o into p. If at the same time sufficient pressure is maintained in tube i, air will flow through q (soldered in screwcap r) into the

agar. The end of needle q is narrowed to a very small opening in order to get the right amount of air in comparison to the amount of agar flowing into the plastic bag (1 ml atmospheric pressure for 5-6 ml of agar or 3 ml per second). Sterilisation of apparatus and agar-methyl-cellulose-nutrient solution mixture is done by autoclaving at 120°C for two hours. Valves a and b (fig. 1) are closed before the container with agar mixture and air filter are placed in the autoclave. The tubes h and i are placed in the loops of the pinchcocks. (Sterilizing these tubes when pinched would spoil them). The screwcap r with canula q is unscrewed from tube o. The open end of the latter is plugged with cotton wool. The amount of air delivered by the canula can be measured after sterilizing without interfering with the sterility of tube o. As the fine opening of q (fig. 2) is liable to be clogged, it is checked before use and adjusted if necessary. For the same reason sterile spare canulae ready fixed in screwcaps must be kept at hand in case a canula becomes irreparably clogged. After checking the amount of air delivered by the canula and before replacing it in tube o, it is surface sterilized with burning alcohol. Contamination of the inside of the canula during the manipulations after the autoclaving, is unlikely.

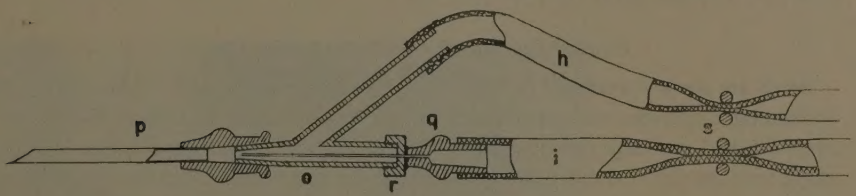

Fig. 2. Apparatus for mixing air and agar, 2/3 natural size. o. Metal tube (fitting into socket of hypodermic needle); p. 2mm hypodermic needle; q. Hypodermic needle adapted for air injection; r. Screwcap for easy replacement of q; s. Two pinchchocks connected so that they open simultaneously; h. Rubber tube conducting agar; i. Rubber tube conducting air.

For cooling and aeration of the agar after sterilisation, the container is shaken gently in a shaking machine, enough to make the agar circulate to bring all of it in contact with air and prevent it from solidifying locally, but not so much that foam is formed, which cannot be used in the bags. During the shaking and the making of the layers in the bags the pressure in the container is kept at 2 atmospheres. When the agar mixture has reached a temperature of about 50°C the making of the layers in the bag can start. The amount of air passing through q is checked, q replaced in o, tubes h and i are replaced in the pinchcocks and valve b is opened.

MAKING THE AGAR LAYERS IN THE BAGS

To make a thin agar layer in a bag the latter is placed in a press made of a piece of stiff metal sheet and a piece of 4 mm perspex sheet of equal size, connected

by a piano hinge (fig. 1, C, n, fig. 5). At the side opposite to the hinge the two plates can be kept together by a clip (fig. 1, m, fig. 5). Near the hinge there is a groove in the perspex just large enough for a 2 mm hypodermic needle. The bag is placed with one corner just under this groove (fig. 6). The corner of the bag in the press, which will lie below the groove in the perspex when the press is closed, is sterilized with alcohol. The hypodermic needle is cleaned with burning alcohol and then inserted in the bag at the sterilized corner (fig. 6). The needle must be well sharpened, otherwise it will stretch the polyethylene before penetration and, instead of slipping between the two layers, it may also pierce the second one. The press is closed and, by opening the pinchcocks, agar with air bubbles is let into the bag (fig. 5). The viscosity and solidifying of the agar between the cold plates prevents the bubbles from ascending quickly so that they stay well dispersed in the agar layer. It is advisible to put the press vertical until the agar has become solid. To prevent micro-organisms from entering the plate, the corner with the puncture is sealed off from the remainder of the bag.

After use, the mixing apparatus (fig. 2) must be cleaned carefully in hot water and the fine canula q blown through and dried. This will keep the aperture of canula q open and free from deformation.

PLANTING SEEDLINGS IN THE AGAR PLATES

Seeds are surface sterilized by treating them for 7 minutes with 0.2 % sublimate containing a spreader and germinated on sterile filterpaper in a Petri dish. Seedlings 0.5 to 1 cm long can be planted. Two cuts (see fig. 7) are made at a right angle near the top of the bag after moistening the area with alcohol for sterilisation of the outer surface. A seedling is then dipped in a saturated solution of bleaching powder (calcium hypochlorite), put on a piece of filter paper for a few seconds and then placed in the agar at the end of the cut, made parallel to the upper edge of the bag, with the cotyledons outside the bag (fig. 7). The cuts are sealed by applying a narrow strip of paraffin wax with low melting point or vaseline-paraffin mixture with a hot needle (fig. 8a). To protect these seals from damage they are covered with strips of adhesive cellophane previously sterilized in ether (fig. 8b). If these strips are put on the unsealed cuts water from the agar often prevents the strips from becoming attached firmly to the polyethylene.

The small opening which remains around the seedling is sealed with some sterile silicone grease.

The bags with growing plants are stored in a slightly slanting position, so as to make all roots grow in one direction against the lower side of the bag where they can easily be observed even with an oil immersion lens.

INOCULATION WITH NEMATODES

Nematodes to be placed in the agar layers are sterilized by putting them in a drop of water containing 0.4 % of streptomycin sulphate (MOUNTAIN, 1959),

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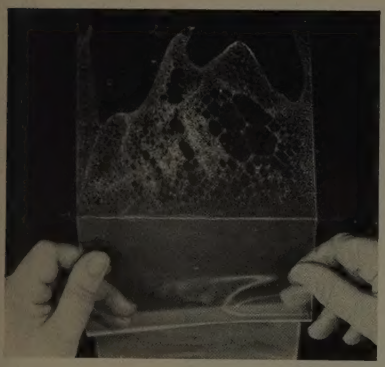

Fig. 3. Removing of air and water from sterilized polyethylene tube.

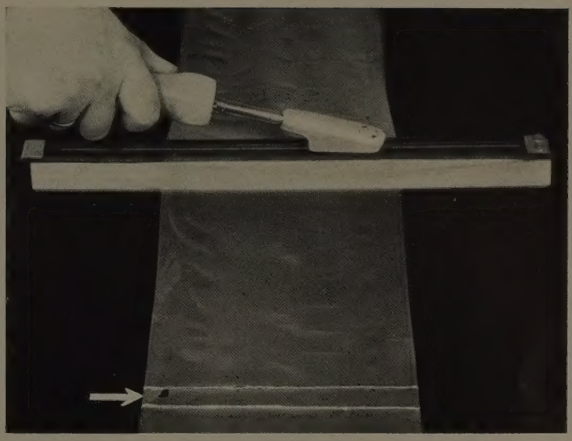

Fig. 4. Sealing apparatus used for cutting polythene tube into closed bags.

Fig. 5. Press used in filling polyethylene bag with mixture of sterile air and agar.

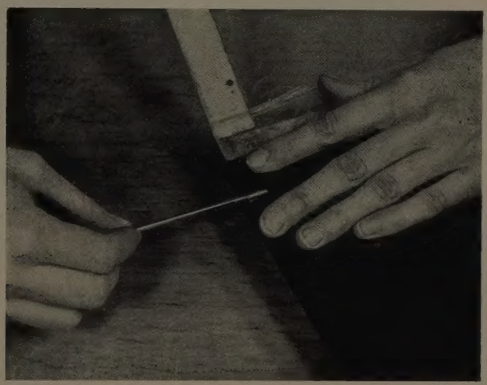

Fig. 6. Inserting 2 mm hypodermic needle into polyethylene bag lying in position on open press.

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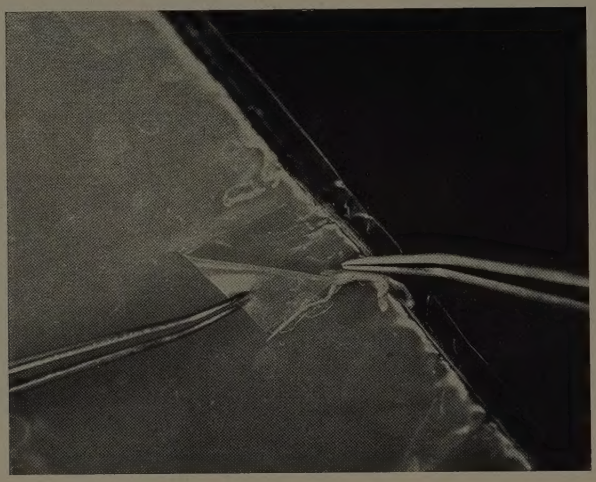

Fig. 7. Inserting a young seedling into a bag.

Fig. 8. a) Sealing of cuts in polyethylene with paraffin wax; b) Covering of seal with adhesive cellophane.

for at least ten minutes. After sterilizing an appropriate area of the bag with alcohol, two cuts are made in the polyethylene as described for the planting of seedlings. Some of the agar below the cuts is removed and the sterile nematodes are placed in the air space made in this way. When a number of nematodes are placed close together in the agar, an air space is necessary to keep the nematodes active. The cuts are sealed as described above. Large numbers of nematodes can be transferred rapidly by placing them first in a small drop of water (or streptomycin sulphate solution) on a freshly made layer of paraffin wax in a watch glass (SEINHORST, 1945). Here a drop does not spread out. If the size of the drop is reduced by sucking off water, it can be brought back to an almost spherical shape by blowing against the edge of the water through a pipette. When the drop is made small enough, practically all nematodes can be taken from it in one movement with a stiff bristle or bamboo splint.

WATERING GROWING PLANTS

As soon as the agar begins to shrink along the roots as a result of water consumption by the plant, sterile water is injected through a hypodermic needle. It is advisable to keep the bags flat for a time, otherwise the water may run off to the lowest place. Sterilizing the bag around the puncture and sealing the hole is done as described before.

ZUSAMMENFASSUNG

Eine verbesserte Methode für die Herstellung von dünnen Agarschichten in Polyäthylensäcken

Nach der beschriebenen Methode können schnell völlig sterile, dünne Agarschichten in Polyäthylensäcken hergestellt werden. Das Agargemisch (2,5% Agar, 0,5% Methylzellulose in Pflanzennährlösung) wird nach Sterilisation und Abkühlung bis etwa 50°C mittels Luftdruck aus dem Sterilisationsgefäss durch einen dünnen Schlauch und eine 2 mm weite Injektionskanüle in die zwischen zwei 1 mm voneinander entfernten Platten liegende inwendig sterilisierte Säcke gepresst. In der Injektionskanüle wird dem Agargemisch etwa 15 Volumprozent steriler Luft in Form von kleinen Bläschen beigemischt.

Das Agar-Methylzellulose-Gemisch nimmt im Gegensatz zu reinem Agar nach Wasserverlust schnell wieder Wasser auf. Durch darauf wachsende Pflanzen dem Nährboden entnommenes Wasser kann deshalb wiederholt ersetzt werden. Es ist notwendig, völlig sterile Keimlinge und Älchen in den Agar zu bringen. Samen können während 7 Minuten mit Sublimat 0,2%, hieraus gewachsene Keimlinge durch Benetzen mit gesättigter Ca-Hypochloritlösung und Nematoden während wenigstens 10 Minuten in 0,4% Streptomyzin-Sulphatlösung genügend desinfiziert werden. Schnittöffnungen die in den äusserlich desinfizierten Polyäthylenbeuteln angebracht werden, um Keimlinge und Nematoden hineinzubringen, werden mit einem in Äther desinfizierten Zellophanklebband verschlossen.

REFERENCES

MOUNTAIN, W. B. & PATRICK, Z. A. (1959). The peach replant problem in Ontario VII. *Canad. J. Bot.* **37**, 459-471.

OUDEN, H. DEN (1958). A new method for culturing plants enabling the observation of nematodes on growing roots. *Tijdschr. Plziekt.* **64**, 269-272.

SEINHORST, J. W. (1945). Een laboratoriummethode voor de bepaling van de vatbaarheid van rogge voor aantasting door het stengelaaltje, *Ditylenchus dipsaci* (Kühn). *Tijdschr. Plziekt.* **51**, 39-52.

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